Discriminative detection of neurotoxins in multi-component samples

A.L. Simonian*, E.N. Efremenko, J.R. Wild
US Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD 21010-5424, USA

Received 24 November 2000; received in revised form 13 April 2001; accepted 13 April 2001

Abstract

In order to develop a new strategy for the detection and discrimination of neurotoxins, the competing anti-acetylcholinesterase activities of mixtures of different neurotoxins were investigated. The combined inhibition effects in mixtures of organophosphates and carbamates (such as paraoxon/carbaryl, diisopropyl fluorophosphate (DFP)/carbaryl, paraoxon/DFP/carbaryl) were different than what was expected from additive effects of single neurotoxins. Mutual interactions of various neurotoxins did lead to competition for acetylcholinesterase (AChE) binding sites, and the overall inhibition effects were not additive but dependent on the types of chemicals present. It was possible to separate the effects of different inhibitors, using a combined recognition/discrimination strategy based on the joint action of acetylcholinesterase and organophosphate hydrolase enzymes. The detection ranges of these integrated biosensors were $10^{-9}$ to $10^{-5}$ M for paraoxon or DFP, and $5 \times 10^{-8}$ to $1 \times 10^{-5}$ M for carbaryl. In addition, it was possible to detect carbaryl concentrations as low as $5 \times 10^{-8}$ M in mixed samples at concentrations up to $10^{-5}$ M of paraoxon and DFP. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Biosensor; Discriminative detection; Acetylcholinesterase inhibitors; Organophosphate hydrolase; Paraoxon; Diisopropyl fluorophosphate; Carbaryl; Coumaphos

1. Introduction

Biosensors have become popular as a tool for environmental monitoring because of their exceptional performance capabilities, including high specificity, rapid response, low cost, and user-friendly operation. Most environmental biosensors are based on inhibition effect of various pollutants (such as heavy metal ions, organophosphorus (OP) neurotoxins, carbamates, etc.) on certain enzymes, such as acetyl or butyryl cholinesterases (AChE and BChE) [1-6]. A number of other enzymes such as urease and glucose oxidase have been also used in inhibition-based biosensors as the target for environmental toxins [7,8]. The main problem for the application of inhibition-based biosensors (specifically cholinesterase-based) is the unpredictable cross-interaction of multiple pollutants presented in the real-world samples. It was shown earlier [9], that the response of a traditional AChE-based biosensor to the samples contained two pesticides, was not simply additive; the “total anti-cholinesterase activity” of mixed samples was very different from the effects of individual concentrations of pesticides. It has been observed that some metals inhibit cholinesterase, both in vivo and in vitro [10]. Other investigations have shown that detergents, combustion-type hydrocarbons, and pulp mills are potential inhibitors of cholinesterases [11].

* Corresponding author. Tel.: +1-979-845-6490; fax: +1-979-845-9274. E-mail address: als@pop.tamu.edu (A.L. Simonian).
Thus, environmental samples, containing multiple unknown pollutants, cannot be adequately analyzed by single cholinesterase-based biosensors; it is necessary to develop new biosensors capable of discriminating between types and classes of inhibitors. One approach to improve the specificity of analysis has been the utilization of a combination of several AChE enzymes from various sources with different substrate specificities [12,13]. While this allows some discrimination between different classes of neurotoxins, it cannot separate the confusing, often contradictory inhibition patterns observed in the presence of different pollutants such as carbamates or heavy metals. Another way to improve specificity of analysis involves the direct detection of neurotoxins based on hydrolysis of organophosphates by enzyme organophosphate hydrolase (OPH) without using cholinesterase [14]. Recently, we have described a new biosensor with a combined biorecognition element based on both AChE and OPH [9]. This approach allows detection of carbamate pesticides in mixtures containing organophosphorus neurotoxins by selectively removing OP neurotoxins. In the present work, the cross-interaction of various pollutants with different chemical natures is examined on AChE/OPH-based biosensor. Paraoxon, DFP, demeton-S, malathion, and coumaphos were evaluated as organophosphorus neurotoxins, and carbaryl was taken as carbamate insecticide of common use.

2. Experimental

2.1. Reagents

Paraoxon (diethyl-p-nitrophenyl phosphate), di-isopropylfluorophosphate (DFP), acetylcholine, and glutaraldehyde were obtained from Sigma (USA); demeton-S (O,O-dimethyl-S-2-ethylthioethyl phosphorothioate), malathion (O,O-dimethyl-S-(1,2-dicarbethoxyethyl) phosphorodithioate), carbosulfan (2,3-Dihydro-2,2-dimethylbenzo[1,2-c:4,5-c']dithiophene), and coumaphos (O,O-Diethyl-O-(chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl) phosphorothioate) were purchased from Chem Service (West Chester, PA, USA). Sodium chloride, glycerol, glycine, and potassium phosphate (monobasic and dibasic) were obtained from Fisher Products, Inc. (USA).

2.2. Enzymes

Acetylcholinesterase (E.C. 3.1.1.7, Type Y1-S, from Electric Eel, 480 U/mg) was obtained from Sigma (USA), organophosphate hydrolase (OPH, E.C. 3.1.8.1, specific activity 14,000 U/mg) was produced from recombinant E. coli strains isolated in our laboratory, and the enzyme was purified according to the methods described earlier [15].

2.3. Immobilization

Silica gel (specific surface of 320 m²/g, density 0.23 g/cm³) was a product of the Yerevan Institute of non-organic Sorbents (Armenia). The AChE and OPH were immobilized on activated silica gel by the methods described earlier [16]. The immobilization for both enzymes (AChE and OPH) was carried out at 4°C for 12 h with gentle stirring. The immobilized biocatalysts, thus, prepared were thoroughly washed with 10 mM PBS buffer, pH 7.4 and stored at 4°C.

2.4. Apparatus and procedures

The combined flat pH electrode FTP-2 was obtained from Lazar, Inc. (USA), and the digital pH meter/Ion Analyzer 350 was from Corning (USA).

A batch mode measurement unit, described in detail previously [17], was developed at the Yerevan Physics Institute (Armenia). The signal was detected by a digital pH meter, registered by computer using ProComm software (Datastorm Technologies, Inc., USA), and analyzed by KaleidaGraph software (Sinegy Software, USA).

In performing the neurotoxic assays, a temperature-controlled (25°C) measurement cell with stir bar was filled with standard buffer (1 mM HEPES, pH 7.8, 100 mM NaCl, 20 mM MgCl₂) and 50 mg (w/w) of silica gel with immobilized AChE. The pH electrode-stopper assembly was plugged carefully to prevent ingress of air bubbles into the measurement cell and 20 μl of 250 mM acetylcholine (final concentration 1 mM) was injected to initiate the reaction; the velocity of ΔpH corresponded to the activity of biocatalyst. In determining AChE inhibition, the biocatalysts were incubated for 15 min with appropriate inhibitor, and then standard measurement procedures were performed to detect residual activity.
The OP neurotoxins were removed from the mixture by pumping solution through column with immobilized OPH for 15 min with flow rate 5 ml/min. Each experimental point was the average of 3–5 measurements.

3. Results and discussion

As shown in our previous publication [9], the response of AChE-based biosensor to mixed samples containing two pesticides of different chemical classes (e.g. paraoxon and carbofuran) was not simply additive, and the experimentally measured concentrations of these pesticides were very different from their real concentrations. It was proposed that in the mixed solution containing both species, the carbamate toxicant with higher affinity to AChE was binding to the active site of enzyme, shielding enzyme active site, and reducing the effect of other toxicants (OP in this case). Thus, leading to faulty analysis. In this study, we investigated the inhibition of AChE in detail with different combinations of inhibitors. The influences of organophosphorus neurotoxins paraoxon, DFP, demeton-S, malathion, and coumaphos were evaluated relative to carbaryl, the representative of carbamate insecticide.

3.1. The effect of single inhibitors

The analysis of AChE inhibition at various concentrations of individual neurotoxins showed that malathion, demeton-S, and coumaphos had the smallest inhibition effect on AChE (Fig. 1). The reduction of AChE activity at concentrations as high as $10^{-5}$ M was <30% for coumaphos and <15% for malathion. The inhibition effectiveness (potency) of the investigated compounds could be ordered in the following sequence: paraoxon > DFP > carbaryl > coumaphos > demeton-S > malathion.

3.2. Combined effects of OPs and carbamate neurotoxins

The inhibition of AChE by OP and carbamates are based on completely different mechanisms. It is well known that carbamates are reversible inhibitors of AChE and they compete with the substrate for enzyme binding site via AChE carbamylation [18]. In contrast, OPs inhibit AChE by blocking the active site serine by

![Fig. 1. Inhibition effects of different neurotoxins. CR: carbaryl; DFP: diisopropyl fluorophosphate; PX: paraoxon; CF: coumaphos; D-S: demeton-S; Mal: malathion.](image-url)
nucleophilic attack to produce a serine phosphoester (via phosphorylation) [18]. These different inhibition patterns could explain the different inhibition effects of binary mixed samples, which contained carbamate and non-carbamate neurotoxins. Fig. 2a summarizes the mutual inhibiting effects of paraoxon/carbaryl mixtures. When the paraoxon concentration was increased at a constant high level of carbaryl concentration (10^{-5} M), the carbaryl effect was not detected. Based on the different molecular mechanisms of AChE interaction with carbamate and organophosphate compounds [9], it might be proposed that when paraoxon occupied the active-site serine of AChE, carbaryl was less able to react with the anionic site.

Another pattern was observed in the case of DFP and carbaryl mixtures. As shown in Fig. 2b, the inhibitory effect of DFP under the same high concentration of carbaryl (10^{-5} M) was less than the same inhibition effect of paraoxon (except PX concentration of 10^{-6} M). In comparison with inhibition effect of DFP alone, the presence of carbaryl considerably decreased the inhibitory effect of DFP over concentration range of 10^{-7} to 10^{-5} M. Thus, carbaryl played the role of protector in this mixture. A possible explanation of this difference in inhibition caused by PX and DFP could be proposed from a comparison of their chemical structures. While interacting with the esteratic site of the AChE active center, paraoxon could protect the anionic site of AChE and prevent the inhibition by carbaryl. However, in the case of DFP, this effect could be greatly reduced because of its smaller molecular structure.

In order to partition the OP and carbamate effects, we utilized the approaches developed in our previous work [9]. All of the samples that contained different combinations of neurotoxins (DFP–CR; PX–CR) were pre-treated with immobilized OPH, which selectively eliminated the inhibition of AChE by organophosphate compounds, according to the following reaction scheme:

After such treatment, the AChE inhibition caused by carbaryl alone was accurately detected (Fig. 2a and b). The subsequent analysis was equivalent to the same concentration of carbaryl (10^{-5} M) (Fig. 1).
3.3. Effects of neurotoxins mixture with the same nature

Another possible combination of neurotoxins in real-world samples may involve the presence of two neurotoxins of the same chemical group, such as paraoxon (P-O) and DFP (P-F). In the presence of high concentrations ($10^{-5}$ M) of both organophosphates, the inhibition was very close to the level observed for paraoxon alone (Fig. 3). Further reduction of paraoxon concentrations in the mixtures with the high constant concentration of DFP ($10^{-5}$ M) led to an increase of the role of DFP in AChE inhibition. The inhibiting effect of mixtures of $10^{-5}$ M DFP and $10^{-8}$ M paraoxon were slightly lower than the inhibition of $10^{-5}$ M DFP alone. This may occur because of competitive interaction of DFP and paraoxon in such mixtures.

Lower concentrations of mixture of paraoxon and DFP cause less inhibition than the individual inhibitors. This effect may be a consequence of two different processes — the inhibition of AChE and competition with other inhibitor for the binding sites of AChE. The culmination of such interaction became the absence of inhibition by mixtures containing $10^{-7}$ and $10^{-8}$ M paraoxon and DFP.

3.4. Effects of mixture with three different neurotoxins

Finally, the inhibition effects of three neurotoxins (paraoxon, DFP, and carbaryl) were investigated. The addition of carbaryl to the mixtures contained paraoxon and DFP resulted in additional AChE inhibition (Fig. 4). High concentrations of all inhibitors completely decreased AChE activity up to 10% (compare with 25% observed after the action of two organophosphates, or 26% revealed after the action of mixture of paraoxon and carbaryl). Decreasing the OP concentrations slightly increased the enzyme’s residual activity. Pre-treatment of the samples with immobilized OPH eliminated the inhibition effect of OPs, and enzyme residual activity approximated to the activity obtained with carbaryl alone (data not shown).

3.5. Discriminative detection strategy

Based on elimination of the influence of organophosphate neurotoxins on the AChE activity in the samples
containing multiple neurotoxins, we propose a new strategy for the discriminative detection of neurotoxins, presented on Fig. 5. On the first step, multi-component sample is analyzed by AChE-based biosensor and data are stored. The second step includes sample treatment by OPH and further analyses by the same AChE-based biosensor. Ultimately, the comparative processing of data analysis will allow the discrimination of OP and non-OP neurotoxins present in the same sample.

3.6. Neurotoxin detection in real-world samples

The capabilities of discriminative detection strategy were applied for the detection of pesticides in potato. Paraoxon and carbaryl was added at $10^{-5} \text{M}$ to each sample. Potato extracts were analyzed at three different concentrations — without dilution, 1/10 and 1/100 dilutions. Samples was incubated with immobilized OPH for discriminative detection. The results presented in Table 1 demonstrated reasonable...
Table 1
Discriminative detection of neurotoxin concentrations in potato

<table>
<thead>
<tr>
<th>Pesticides</th>
<th>Residual activity (%)</th>
<th>Diluted potato extract × 10</th>
<th>Diluted potato extract × 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato extract without pesticides</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Potato extract + 10⁻⁵ M paraoxon</td>
<td>31</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Potato extract + 10⁻⁵ M paraoxon + carbaryl</td>
<td>29 (10⁻⁵ M carbaryl)</td>
<td>27 (10⁻⁶ M carbaryl)</td>
<td>37 (10⁻⁷ M carbaryl)</td>
</tr>
<tr>
<td>Potato extract + carbaryl</td>
<td>40 (10⁻⁵ M carbaryl)</td>
<td>65 (10⁻⁶ M carbaryl)</td>
<td>87 (10⁻⁷ M carbaryl)</td>
</tr>
<tr>
<td>Potato extract + 10⁻⁵ M paraoxon + carbaryl, pre-treated with OPH</td>
<td>47 (10⁻⁵ M carbaryl)</td>
<td>68 (10⁻⁶ M carbaryl)</td>
<td>91 (10⁻⁷ M carbaryl)</td>
</tr>
</tbody>
</table>

3.7. Mathematical model

To separate the effects of each inhibitor in the residual activity of AChE in multi-component samples, we applied the methods of partitioning analysis to calculate the impact of individual neurotoxins from the total inhibition values. In accordance with complete factors analysis (three factors with two levels of variation) the data on AChE inhibition were statistically evaluated and the empirical equation of regression was obtained:

\[ Y = 41.28 - 19.49X_1 - 6.41X_2 - 5.52X_3 + 2.89X_1X_2 + 2.46X_1X_3 + 8.74X_2X_3 - 13.21X_1X_2X_3 \]

where \( Y \) is the residual activity of AChE in presence of different neurotoxins, and \( X_1, X_2, X_3 \) the concentrations of paraoxon, DFP and carbaryl, respectively.

This equation reflects the contribution of each factor and their mixtures to the final AChE inhibition effect. By taking into account the value of the coefficients for each term, it can be concluded that the negative coefficient has more value than the inhibiting effect on the AChE activity revealed by the corresponding individual neurotoxins. The positive value of all coefficients characterized by a two factors interaction shows the existing competition between inhibitors, which resulted in a reduction of total inhibition effect. This equation indicates that the strongest inhibitor among three investigated neurotoxins is paraoxon.

4. Conclusion

The data presented above demonstrated that samples contained more than one inhibitor could not be adequately analyzed by AChE inhibition-based biosensors alone. The same experimental data cannot distinguish between the presence and the absence of low or high concentrations of inhibitors in samples containing more than one type inhibiting neurotoxin. In these cases, when there are two organophosphate neurotoxins in the sample, the data can reflect the presence of only one of them, or may even demonstrate the great reduction of inhibition because of the competition that exists among the inhibitors. In the presence of neurotoxins from different chemical groups, such as organophosphates and carbamates, the data may be masked or greatly underestimated compared to the presence of only one inhibitor. It also may indicate the presence of inhibitors at much lower concentrations because of the “protection” of the AChE by carbamate inhibitor. Thus, it is impossible to have an adequate interpretation of the results obtained after the AChE exposure with unknown sample containing more than one inhibitor. We demonstrated the feasibility of eliminating the organophosphate neurotoxins from the different multi-component inhibitor combinations via sample pre-treatment with immobilized forms of OPH. Because of such manipulation, the inhibiting influence of non-OP neurotoxins on the AChE can be separated and its true concentrations may be determined.

Acknowledgements

This research was supported in part by Sandia National Laboratories, CA (Grant #LG-3101, JRW and
ALS), US Army Medical Research and Materiel Command (JRW), and CRDF grant (ENE). We thank Evgenia Rainina for fruitful discussion.

References